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Preparation of *Terrific Broth and LB Agar*

1. Weight 35 grams of LB Agar (Sigma-Aldrich) for each liter of media.
2. Weight 42.5 grams of Terrific Broth (Sigma-Aldrich) for each liter of media.
3. Dilute in Erlenmeyer flasks with the adequate amount of distilled water.
4. Heat in constant agitation.
5. Place the flasks in the autoclave with autoclave tape.
6. Set te autoclave until it reaches 120 °C and 15 PSI.
7. Store both the Broth and Agar at 4 °C.

Preparation of Antibiotic stocks

Cloramphenicol Stock:

1. Weight 10 mg of lyophilized antibiotic for each ml of stock solution.
2. Dissolve in ethanol and mix gently (It is possible to use Vortex)

Kanamicin Stock:

1. Weight 50 mg of lyophilized antibiotic for each ml of stock solution.
2. Dissolve in distilled water and mix gently (It is possible to use Vortex).

Ampicilin Stock:

1. Weight 100 mg of liophylized antibiotic for each ml of stock solution.
2. Dissolve in distilled water and mix gently (It is possible to use Vortex).
3. For each 2 ml of media use 1 ul of stock antibiotic.
1. Therefore the working concentration for each antibiotic (for use in and Agrobacterium tumefaciens and E. coli) will be:Cloramphenicol: 5ul/ml
2. Kanamicin: 25 ul/ml
3. Ampicilin: 50 ul/ml

Hannahan method for Competent Cells

1. Inoculate 250 Ml of SOB medium with a 1 Ml vial of seed stock
2. Incubate the culture at 20°C in a shaking incubator for ~16 h. From time to time, check the optical density of an aliquot of the culture at 600 nm. Do not allow the culture to grow to an OD600 > 0.26.
3. Transfer the culture to prechilled, flat-bottomed centrifuge bottles and centrifuge for 10 min at 4°C.
4. Remove as much of the supernatant as possible by pouring, followed by gentle gentle aspiration through a wide-bore pipette
5. Gently resuspend the cells by adding 20 mL of ice-cold CCMB80 buffer and swirling the contents of the centrifuge bottle. When the pellet is reasonably well suspended, add another 60 mL of ice-cold CCMB80 buffer and swirl again. If the suspension is still lumpy, disperse the lumps by very gentle up-and-down pipetting. Then, incubate the suspension for 20 min in ice.
6. Centrifuge the suspension for 10 min at 4° C. Read the OD at 600 nm.
7. Remove the supernatant as before and resuspend the pellet in 10 mL of ice-cold CCMB80.
8. Transfer 200 uL of SOB to a plastic tube. Add 50 uL of the E.coli suspension and mix gently, avoiding air bubbles.
9. Calculate the amount of CCMB80 buffer required to adjust the OD of the remainder of the bacterial suspension to between 1.0 and 1.5. Add the required amount of ice cold CCMB80 to the suspension.
10. Incubate the diluted suspension for 20 min on ice.
11. Dispense 50-uL aliquots into prechilled 2-mL screw-top freezing vials.
12. Aliquot 1-mL quantities into 2.5 mL cryotubes. Insert the tubes into a plastic zipper bag and immerse the bag for 5 min in a dry-ice/ethanol bath.
13. Transfer the bag and its contents to a -80°C freezer.

Heat Shock Transformation of *E.coli*

Note: Never vortex competent cells. Mix cells by gentle shaking.

1. Thaw competent cells on ice.
2. Place 20 ul of cells in a pre-chilled Eppendorf tube.

   For an Intact Vector: Add 0.5 ul or less to the chilled cells

   For a Ligation Product: Add 2-3 ul to the chilled cells.
3. Mix gently by flicking the tube.
4. Chill on ice for 10 minutes. (Optional)
5. Heat shock at 42 °C for 50 seconds.
6. Incubate on ice for 2 minutes.
7. Add 200 ul LB, Terrific or SOC medium and recover the cells by shaking at 37 °C.
8. The recovery time varies with the antibiotic selection.

   Ampicillin: 15-30 minutes

   Kanamycin or Spectinomycin: 30-60 minutes

   Chloramphenicol: 60-120 minutes
9. Plate out the cells on selective LB. Use glass beads to spread the cells. The volume of cells plated depends on what is being transformed.

   For an Intact Vector: High transformation efficiencies are expected. Plating out 10 ul of recovered cells should produce many colonies.

   For a Ligation Product: Lower transformation efficiencies are expected. Therefore you can plate the entire 200 ul volume of recovered cells.
10. Incubate at 37 °C. Transformants should appear within 8 – 16 hrs.

Plasmid purification by Miniprep

1. Centrifuge an eppendorf tube of 1.5ml with L. Plantarum culture at 12000 rpm in the mini-spin for 1 minute, two times.
2. Discard the supernatant and resuspend the tube's content with more L. Plantarum culture in MRS broth.
3. Centrifuge the eppendorf tube for 15min at 6000 rpm.
4. Discard the supernatant.
5. Resuspend the pellet in 250ml Resuspension buffer.
6. Add 250ml of Lysis Buffer.
7. Gently mix the tube carefully inverting it 5 times carefully.
8. Add and mix softly 350ml of Precipitation Buffer, inverting the tube.
9. Centrifuge it at 12000 rpm for 10 minutes.
10. Transfer the supernatant into a spin column inside a washtube.
11. Centrifuge it at 12000 rpm for a minute.
12. Discard the supernatant and add 500 ml of Wash Buffer with ethanol (w10) to the column.
13. Incubate it for one minute at room temperature.
14. Centrifuge the column at 12000 rpm for 1 minute.
15. Discard the liquid from the washtube and place the column inside the tube.
16. Add 700ml of Wash Buffer W9 with ethanol to the column.
17. Centrifuge the column with the washtube at 12000 rpm for 1 minute.
18. Discard the liquid from the washtube.
19. Centrifuge the column with the washtube at 12000 rpm for 1 minute.
20. Discard the liquid from the washtube.
21. Place the column inside an eppendorf tube of 1.5ml.
22. Add 75ml of preheated TE Buffer at the center of the column.
23. (Warm the TE Buffer previously in water bath at 65ºc-70ºc for 3 minutes).
24. Incubate the column for 1 minute at room temperature.
25. The column was centrifuged at 12000 rpm for 2 minutes.
26. (The eppendorf tube contains the purified plasmid).

Plasmid purification by Midiprep

1. Harvest the cells by centrifugation for 10 min at 12000 rpm in the mini-spin. Discard the supernatant.
2. Resuspend the pelleted cells in 2 mL of Resuspension Solution auditioned with RNase solution. The bacterial pellet should be resuspended by vortexing or pipetting up and down until no cell clumps remain.
3. Add 2 mL of Lysis Solution and mix gently by inverting the tube 4-6 times until the solution becomes viscous and slightly clear. Incubate for 3 min at room temperature.
4. Add 0.5 mL of the Endotoxin Binding Reagent. Mix immediately by inverting the tube 5-8 times.
5. Incubate for 5 min at room temperature. Note. After the addition of the Neutralization Solution and Endotoxin Binding Reagent it is important to mix gently, but thoroughly, to avoid localized precipitation of bacterial cell debris. The neutralized bacterial lysate should appear cloudy and contain white precipitate.
6. Add 3 mL of 96% ethanol. Mix immediately by inverting the tube 5-6 times.
7. Centrifuge for 10 min at 4,000-5,000 rpm to pellet cell debris and chromosomal DNA.
8. Transfer the supernatant to a 15 mL tube (not provided) by decanting or pipetting. Avoid disturbing or transferring the white precipitate.
9. Add 3 mL of 96% ethanol. Mix immediately by inverting the tube 5-6 times.
10. Transfer part of the sample (~ 5.5 mL) to the supplied column pre-assembled with a collection tube (15 mL). Be careful not to overfill the column. Centrifuge for 3 min 10000 rpm in the mini-spin. Discard the flow-through and place the column back into the same collection tube.
11. Repeat the last step to process any remaining lysate through the purification column.
12. Add 4 mL of Wash Solution I (diluted with isopropanol) to the purification column. Centrifuge for 2 min. at 4000 rpm in a swinging bucket rotor. Discard the flow-through and place the column back into the same collection tube.
13. Add 4 mL of Wash Solution II (diluted with ethanol) to the purification 6 column. Centrifuge for 2 min. at 5000 rpm. Discard the flow-through and place the column back into the same collection tube.

14. Repeat the column wash with Wash Solution II
15. Centrifuge for 5 min at 3,000 × g in a swinging bucket rotor to remove residual wash solution. Discard the collection tube containing the flow-through.
16. Transfer the column into a fresh 15 mL collection tube (provided). Add 0.35 mL of the Elution Buffer to the centre of the purification column membrane. Incubate for 2 min at room temperature and centrifuge for 5 min at 5000 rpm to elute plasmid DNA.
17. Discard the purification column. Use the purified plasmid DNA in downstream applications or store DNA at -20°C.

Electrophoresis Agarose Gel

1. For each 100 ml of electrophoresis gel, weight 1 g of agarose.
2. Dissolve in the adequate amount of 1x TAE Buffer.
3. Heat in constant agitation. It could be done in microwave by heating in short intervals and agitating manually.
4. When completely dissolved let the flask cool a little then and 1.2 l of ethidium bromide 5 % p/p for each 50 ml of gel.
5. Agitate until it's completely dissolved. If not used immediately store at 4 °C.
6. Pour the agarose into a gel tray with the suitable well comb in place (pour slowly to avoid bubbles which will disrupt the gel).
7. Place newly poured gel at room temperature for 20-30 minutes, until the gel has completely solidified.
8. Once solidified, remove the comb and place the gel into the electrophoresis unit (gel box).
9. Fill the gel box with 1x TAE buffer.
10. Load GeneRuler 1Kb Plus DNA Ladder 0.1 ug/ml weight one lane of the gel.
11. Carefully load your samples into the additional wells of the gel, carefully mixed with 6X DNA Loading Dye.
12. Run the gel at 50-150V until the dye line is approximately 75-80% of the way down the gel (20-45 min).
13. Use an UV light transilluminator to observe the DNA fragments on the gel.

Restriction Digest

1. Keep all enzymes and buffers used on ice.
2. Thaw NEB Buffer 2 and BSA in room temperature water. Mix by shaking the tubes, and flick/spin them to collect the liquid at the bottom of the tube.
3. Add 250ng of DNA to the appropriately labelled tube. Add distilled water to the tubes for a total volume of 16ul in each tube.
4. Pipet 2.5ul of NEB Buffer 2 to each tube.
5. Pipet 0.5ul of BSA to each tube.
6. In the Part A tube: Add 0.5ul of EcoRI, and 0.5ul of SpeI.
7. In the Part B tube: Add 0.5ul of XbaI, and 0.5ul of PstI.
8. In the pSB1C3 tube: Add 0.5ul of EcoRI, 0.5ul of PstI, and 0.5ul of Dpn1.
9. The total volume in each tube should be approximately 20ul. Mix well by pipetting slowly up and down. Spin the samples briefly to collect all of the mixture to the bottom of the tube.
10. Incubate the restriction digests at 37°C for 30 minutes, then 80°C for 20 minutes. We use a thermal cycler with a heated lid.
11. Use ~2ul of the digest (25ng of DNA) for ligations.

	Part A	Part B	linearized plasmid backbone
DNA	250ng	250ng	250ng (10ul @ 25ng/ul)
dH2O	adjust to 16ul	adjust to 16ul	6ul
NEB Buffer 2	2.5ul	2.5ul	2.5ul
BSA	0.5ul	0.5ul	0.5ul
Enzyme 1	0.5ul EcoRI	0.5ul XbaI	0.5ul EcoRI
Enzyme 2 Cell:1	0.5ul SpeI	0.5ul PstI	0.5ul Pst1
Enzyme 3			0.5ul DpnI

Parts Ligation

1. Add the following reaction components in the order listed below, centrifuge briefly, and incubate at room temperature for 5 min.
2. Following digestion, remove 5' phosphates from vector using an alkaline phosphatase. Do not dephosphorylate the gBlocks Gene Fragment.
3. Following dephosphorylation, gel purify the vector to reduce background and eliminate enzymes, and quantify.
4. Following digestion, purify gBlocks Gene Fragment insert with QIAquick column, and quantify. The gBlock Gene Fragment should not be dephosphorylated.

Product	Amount
Linearized Vector	50 ng
gBlocks Gene Fragment	3-5X molar excess
2X Quick Ligase Buffer	10 ul (each)
Quick DNA Ligase	To 1ul
DNase- and RNase- free water	For a final volume of 20 uL

Cohesive-end restriction cloning of gBlocks ™ Gene Fragments

1. Add the reaction components to the digestion as in the table below
2. Following digestion, remove 5' phosphates from vector using an alkaline phosphatase. Do not dephosphorylate the gBlocks Gene Fragment.
3. Following dephosphorylation, gel purify the vector to reduce background and eliminate enzymes, and quantify.
4. Following digestion, purify gBlocks Gene Fragment insert with QIAquick column, and quantify. The gBlock Gene Fragment should not be dephosphorylated.

Product	gBlock	Vector
DNA	100ng	600ng
10x Buffer	3ul	3ul
Restriction Enzyme	1ul (each)	1ul (each)
Nuclease free water	To 20ul	To 30ul